Culture and characterisation of canine mitral valve interstitial and endothelial cells

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Abstract: Valve interstitial cells (VICs) have an important role in the aetiopathogenesis of myxomatous mitral valve disease (MMVD) in the dog. Furthermore, there is evidence that valve endothelial cells (VECs) also contribute to disease development. In addition to examining native valve tissue to understand MMVD, another strategy is to separately examine VIC and VEC biology under in vitro culture conditions. The aim of this study was to isolate and characterise canine mitral VICs and VECs from normal dog valves using a combination of morphology, immunohistochemistry and reverse transcription PCR (RT-PCR).

Canine mitral VECs and VICs were isolated and cultured in vitro. The two cell populations exhibited different morphologies and growth patterns. VECs, but not VICs, expressed the endothelial markers, platelet endothelial cell adhesion molecule (PECAM-1 or CD31) and acetylated low density lipoprotein (Dil-Ac-LDL). Both VECs and VICs expressed vimentin and embryonic non-smooth muscle myosin heavy chain (SMemb), an activated mesenchymal cell marker. The myofibroblast marker, alpha smooth muscle actin (α-SMA), was detected at the mRNA level in both VEC and VIC cultures, but only at the protein level in VIC cultures. The morphological heterogeneity and expression of non-endothelial phenotypic markers in VEC cultures suggested that a mixture of cell types was present, which might be due to cell contamination and/or endothelial mesenchymal transition (EndoMT). The use of a specific endothelial culture medium for primary VEC cultures enhanced the endothelial properties of the cells and reduced α-SMA and SMemb expression.

Culture and characterisation of canine mitral valve interstitial and endothelial cells

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Abstract

Valve interstitial cells (VICs) have an important role in the aetiopathogenesis of myxomatous mitral valve disease (MMVD) in the dog. Furthermore, there is evidence that valve endothelial cells (VECs) also contribute to disease development. In addition to examining native valve tissue to understand MMVD, another strategy is to separately examine VIC and VEC biology under in vitro culture conditions. The aim of this study was to isolate and characterise canine mitral VICs and VECs from normal dog valves using a combination of morphology, immunohistochemistry and reverse transcription PCR (RT-PCR).

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Introduction

Myxomatous mitral valve disease (MMVD) is characterised by extensive changes to the valve extra cellular matrix (ECM), resulting in valve distortion and mechanical instability, and leading to valve insufficiency and mitral regurgitation (Black et al., 2005; Hadian et al., 2007; Aupperle et al., 2009, 2010; Han et al., 2010, 2013b). The mechanisms that lead to valve degeneration are not fully understood, but are presumed to involve changes in valve interstitial cell (VIC) and endothelial cell (VEC) phenotype and function (Corcoran et al., 2004; Disatian et al., 2008; Han et al., 2008, 2013a). Endothelial changes are characterised by detachment, denuding and apoptosis with splitting of the basement membrane and increased expression of basement membrane proteins, including laminin (Aupperle et al., 2009; Han et al., 2013a). VICs show an activated myofibroblast phenotype and their numbers decline in the overly myxomatous areas and proliferate close to the valve surface, most noticeably where there has been endothelial damage (Disatian et al., 2008; Han et al., 2008, 2013a).

VICs are the cells contributing to ECM production and remodelling. Their synthetic activity is driven mainly by the transforming growth factor beta (TGF-β) cytokine superfamily, but is also thought to be modified by signals from the endothelial cells (Olsen et al., 2003; Disatian et al., 2010; Aupperle and Disatian, 2012; Orton et al., 2012; Han et al., 2013a). This VEC-VIC interaction has led to the hypothesis that VEC activation is fundamental to valve matrix homeostasis and that MMVD may be driven by mechanisms involving VEC damage and leading to aberrant matrix remodelling.

Future examination of MMVD will require a greater understanding of VEC-VIC interactions and one approach is to do so using in vitro models. Canine mitral VICs have been previously isolated and cultured using a primary explant technique which favours

culturing migratory cells only, but there was limited phenotypic characterisation, and there are no reports of successful cultures of canine VECs (Heaney et al., 2009). The aims of this study, therefore, were (1) to develop robust methods for the culture and phenotypic characterisation of canine mitral VICs and VECs based on a reported protocol for porcine aortic valve cell isolation (Gould and Butcher, 2010), and (2) to examine canine mitral valve cell biology under the culture conditions. Furthermore, as it is recognised to be difficult to maintain endothelial cell phenotype in VEC culture, different culture protocols were evaluated.

Materials and methods

Cell isolation and culture

Cells were isolated from 12 healthy canine mitral valves showing no MMVD evidence on gross morphological inspection. The dogs were of different breeds, aged 2-5 years and with equal sex distribution. All dogs were euthanased for reasons other than cardiac disease. Samples were collected with full owner consent and sampling conformed to institutional and national ethical guidelines.

Mitral valves were removed within minutes of euthanasia under sterile conditions, rinsed in cold sterile phosphate buffered saline (PBS) and placed on ice until transferred to a tissue culture laminar hood. Excess annular tissue and chordae tendineae were removed, and samples were placed in 35 mm petri dishes and incubated with 5 mL pre-warmed collagenase solution (600 Units/ml) in 5% CO_2 for 10 min at 37 ºC. VECs were removed by gently rotating a dry sterile swab over the surface of the leaflet and occasionally dabbing the swab into the collagenase solution. The collagenase solution was aspirated and the suspended cells pelleted by centrifuging at 1000g for 5 min at room temperature. The supernatant was

aspirated and the cells were re-suspended in advanced DMEM/F-12 medium (Life Technologies) with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin and 1% L-glutamine (AF-12). Cells were centrifuged, re-suspended again and seeded on culture flasks or plates pre-coated with 2% gelatin and incubated at 37 °C in 5% CO_2. After VEC collection, tissue was placed in a 15 mL conical tube with 10 mL collagenase solution (600 U/mL) and incubated for 18 h at 37 °C in 5% CO_2. After digestion, the tissue was homogenized in the collagenase solution. The released cells were then seeded onto flasks and cultured as described for VECs in AF-12 medium.

Media changes occurred at 2 day intervals for both populations. At confluence, cells were passaged by either trypsinization (0.05% - 1% Trypsin /EDTA) or by using 1 x TrypLE Express (Life Technologies). In general, VECs were expanded up to passage three and VICs were cultured up to passage 8.

Comparison of two culture media for canine mitral VECs

To evaluate the effect of specific endothelial culture medium on primary VEC properties, a canine endothelial basal medium kit (Cn 211k-500, Cell Application; EBM) was compared with the AF-12 medium for VECs. VEC preparations (at passages 1-2) from different dogs (n = 3) were used for the medium comparison experiment. Prior to the medium switch, cells were stored in freezing medium (70% AF-12 medium/20% FBS/10% dimethylsulphoxide (DMSO; Invitrogen)) and cryopreserved in -150 °C freezer or in liquid nitrogen freezer. Cells were revived in a 37 °C water bath, and were washed with pre-warmed AF-12 medium by centrifuging. After removal of the supernatant, the cells were re-suspended in canine EBM and seeded onto culture plates pre-coated with 2% gelatin and incubated at 37 °C in 5% CO_2. Control cultures were maintained in AF-12 for the same VEC preparation.

VEC cultures in EBM and AF-12 were harvested at confluence and evaluated for endothelial and mesenchymal marker expression.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from both cell types using the RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. Extracted RNA was treated with RNase-free DNase I and concentration of RNA was measured using NanoDrop (Thermal Scientific). RNA was diluted using nuclease-free water (Qiagen) and was denatured at 65 °C for 5 min, then placed immediately on ice. Complementary DNA was synthesized using an Omniscript Reverse Transcription Kit (Qiagen) at 40 °C for 1 h.

The following genes (Table 1) were selected to characterise the VECs and VICs: *CD31* and *von Willebrand Factor* (*vWF*) (endothelial markers); *alpha-smooth muscle actin* (*α-SMA*), *embryonic form of non-smooth muscle myosin* (*SMemb*) and *transgelin* (*SM22*) (activated VIC (aVIC) markers); *vimentin* (mesenchymal origin cells). *GAPDH* was used as a housekeeping gene. The primers of *vWF-1*, *α-SMA* and *GAPDH* were kind gifts (Hannah Hodgkiss-Geere, the University of Edinburgh), and vWF3 primer sequence was derived from Fulton et al. (2000). All other primers were originally designed using Primer 3 Input v.4.0 and nucleotide sequences of the above markers were obtained from the NCBI Gene Bank or Ensembl databases.

PCR amplification used the Gotaq PCR Core System (Promega). Initial denaturation at 95 ℃ for 5 min was followed by 30 cycles of 95 ℃ for 1 min, 58-61 °C for 1 min, 72 ℃ for 1 min and a final extension at 72 °C for 10 min. PCR products were analysed using a

standard 2% agarose gel electrophoresis. Molecular Imager Gel Doc system (Bio-Rad) was used to visualize the results after electrophoresis.

Immunocytochemistry

The cell suspension (200 µL) was added to duplicate BD Falcon Culture slides, pre-coated with 2% gelatin for VECs, at a density of 1 x 10^4 cell/well and incubated in 5% CO_2 at 37 °C for 24-48 h. Slides were directly fixed with acetone for 10 min at -20 ^oC, washed twice in PBS, incubated for 1 h in blocking buffer (10% goat serum/0.1% Tween 20/PBS) and then overnight in a humidity chamber at 4 °C with primary antibodies against CD31, vimentin, SMemb and α-SMA (Table 2). Slides were washed twice with PBS and incubated with 100 µL goat anti-rabbit IgG or goat anti-mouse IgG secondary antibody for 1 h at room temperature in a dark humid chamber. The slides were then washed three times in PBS, 4',6-diamidino-2-phenylindole (DAPI) mounted (Vectashield Mounting Medium,Vector Inc), and examined using a Leitz fluorescence microscope (Leica).

DiI- acetylated-low density lipoprotein (DiI-Ac-LDL) labelling

Acetylated low density lipoprotein conjugated with fluorescence dye 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was used to detect endothelial cells. The cell culture protocol prior to labelling was the same as that described for immunocytochemistry. DiI-Ac-LDL (200 µL; 5 µg/mL; BT902, Biomedical Technologies) reagent was added to one cell chamber and incubated for 4 h in 5% CO_2 at 37 °C. Slides were washed three times with PBS and were examined by fluorescence and bright field microscopy.

Image capture and processing

Cell morphology was observed by light microscopy during the culture period and assessed for cell morphology and confluence. Representative images were captured using a camera connected Zeiss Axiover 40 microscope. Immunofluorescence and DiI-Ac-LDL fluorescent channel images were captured using a Leica Firecam (Leica). For optimal visualization of the fluorescence signals, the antibody and DAPI staining images were converted into grayscale with Image J software. Original antibody and DAPI channel colour images were merged using Adobe Photoshop CS6 software (Adobe System). For DiI-Ac-LDL labelling, bright field images were taken from the same sampled area demonstrating cell distribution and were converted into grayscale images with ImageJ software.

Results

Cell culture morphology and growth

By using current collagenase digestion techniques, VEC and VIC were successfully isolated from the same mitral valve (n = 12) and grew well in all sub-cultures. The two culture types showed differences in cell morphology and growth pattern.

Endothelial colony-forming cell clusters were randomly distributed in VEC culture flasks 1-2 days post primary isolation, and clustering promoted outgrowth. Cells had cobblestone morphology typical of endothelial cells in culture, and most noticeably at confluence (Fig.1). Confluence of passage 0 VECs was usually reached within 6-8 days in one T80 flask when most cells exhibited growth contact inhibition and formed a monolayer. Some cell polymorphism was also noted with spindle shapes and multiple cytoplasmic extensions, which increased in numbers with increasing passages.

Cultured VICs typically had a spindle shape reminiscent of a fibroblast-like morphology, although heterogeneous morphologies were also observed, and the cells proliferated readily in culture (Fig.1). At confluence, the cells tended to form a whorl-like pattern with multiple layers and did not exhibit growth contact inhibition (Fig. 1). Primary passage 0 VICs usually reached at confluence in a T80 flask within 3 days.

VEC phenotypic characterization

Phenotypic characterization was performed on 3-4 VEC cell lines (at passages 2-3) derived from different dogs. By RT-PCR, VECs expressed CD31, vimentin, as well as α-SMA, SMemb and SM22 (Fig. 2). Expression of vWF was not apparent in canine mitral VECs, and this finding was consistently observed with three different vWF primer pairs. All vWF primer pairs were validated by probing cDNA of two endothelial nature cell lines (one human umbilical vascular endothelial cell line and one canine haemangiosarcoma cell line), in which the expression was clearly identified (data not show).

By immunofluorescence, CD31 was identified in VECs, particularly at cell-cell junctions (Fig. 3). VECs also extensively expressed vimentin and SMemb in the cytoplasm (Fig. 3), while α-SMA expression was limited and only detected in a few cells from one culture. The endothelial identity of the VEC culture was also confirmed by DiI-Ac-LDL uptake (Fig. 3).

Effects of culture media on VEC culture characteristics

Replacing standard culture medium (AF-12) with EBM helped to maintain a more endothelial phenotype as determined by morphological assessment. Cells in both media showed Dil-Ac-LDL up-take and CD31 expression, but staining was more intense in cells

cultured with EBM (Fig. 4). At the transcriptional level, higher CD31 expression was observed in EBM cultures compared with AF-12 cultures, while α-SMA and SMemb expression was reduced in the EBM cultures compared to AF-12 cultures (Fig. 5).

VIC phenotypic characterization

Phenotypic characterization was performed on 3-5 VIC cell lines (at passages 4-7) derived from different dogs. By RT-PCR, α-SMA, vimentin, SM22 and SMemb were expressed in VIC cultures in contrast to CD31 (Fig. 2). By immunofluorescence, VIC cultures were also consistently negative for CD31 expression (Fig. 6). Similar to VECs, VICs showed extensive cytoplasmic expression of vimentin and SMemb. α-SMA expression was observed in the cytoplasm, although the expression level varied between different cultures, with an estimated average of 10% cell expression. No VIC cultures showed DiI-Ac-LDL uptake (Fig. 6).

Discussion

VICs have been previously cultured from dog mitral valves from normal dogs and in one study from dogs with mild MMVD (Heaney et al., 2009; Waxman et al., 2012). In the study by Heaney et al. (2009), cultures were initiated up to 36 h after death (Heaney et al., 2009). Furthermore, their protocol favoured migratory cells, resulting in suboptimal VIC isolation and culture (Taylor et al., 2000). Waxman et al. (2012) isolated mitral VICs using the same collagenase digestion technique as in our study, but from a limited number of laboratory Beagles that were healthy or with mild MMVD. VIC characteristics in a standard tissue culture system, however, were not evaluated. To date there are no reports of culture and characterisation of VECs collected from canine valves immediately after death.

In the current study, canine mitral VECs and VICs were successfully isolated from the same subjects, cultured and characterized. Relatively pure cell populations were obtained and there was differential expression of a range of known mitral valve cell phenotypic markers. This provides fundamental information on canine mitral valve cell biology, which will benefit mitral valve disease research.

VECs are known to express CD31 and to show selective uptake of DiI-Ac-LDL through endocytosis. These properties allow confirmation of an endothelial origin (Cuy et al., 2003; Gould and Butcher, 2010). The lack of expression of vWF was unexpected, but could be due to a tissue-specific heterogeneous expression of vWF, as has been previously observed in endothelium from different anatomic sites (Pusztaszeri et al., 2006). A previous report on porcine mitral VECs also found vWF expression to be weak at the transcriptional level (Flanagan et al., 2006). The majority of VECs exhibited typical cobblestone morphology, but cellular pleomorphism was also present with spindle-shaped cells reminiscent of interstitial cells and fibroblasts. This may be explained by valve-side heterogeneity (as found in aortic valve VECs), VIC contamination or endothelial mesenchymal transition (EndoMT) to a VIC phenotype (Simmons et al., 2005; Bischoff and Aikawa, 2011). In the current study, the mRNA expression of aVIC markers (i.e. SMemb, SM22 and α-SMA) in VEC cultures suggests the presence of VIC contamination and/or EndoMT in the culture system. VIC contamination is a common issue in primary VEC cultures when using enzymatic digestion (Butcher and Nerem, 2007; Cheung et al., 2008). However, cell contamination cannot fully explain the aVIC characteristics in the VEC cultures, particularly the extensive protein expression of the activated mesenchymal marker SMemb in VEC. To our knowledge, this is the first study showing SMemb expression in adult VECs, which suggests that VECs in the

current culture system are in an activated state and have embryonic cell potential reminiscent of EndoMT.

EndoMT does involve increased mesenchymal and decreased endothelial marker expression (Hoerstrup et al., 1998; Cheung et al., 2008; Bischoff and Aikawa, 2011). Such phenotypic transformation of VECs has been suggested to be similar to what occurs during valvular development (Person et al., 2005; Butcher and Markwald, 2007; Butcher and Nerem, 2007; Bischoff and Aikawa, 2011). The complexity of the transition from cardiac endothelium to interstitial cells involves multiple signalling pathways, but particularly endothelial growth factors and member of the TGF-β superfamily (Armstrong and Bischoff, 2004). The possible EndoMT potential in adult valve cells has been examined previously (Paruchuri et al., 2006; Paranya et al., 2001; Mahler et al., 2013). It has been hypothesized to be one of the key remodelling mechanisms in heart valve diseases, where a subset of adult VECs possess foetal cell characteristics similar to progenitor cells, and can replenish the VIC population when needed, for example during disease or remodelling process (Paruchuri et al., 2006; Liu et al., 2007; Bischoff and Aikawa, 2011).

Due to cell contamination and/or in vitro differentiation, the primary VECs were less stable particularly in long term cultures. Cell purification techniques and special culture medium favouring endothelial growth are potential solutions to increase VEC purity (Hoerstrup et al., 1998; Cheung et al., 2008; Gould and Butcher, 2010). In the current study, these two methods were evaluated separately. Trial cell sorting experiments were performed on the VECs from one canine mitral valve (data not shown). By using CD146 and flow cytometry (Wills et al., 2009), over 75% of positive cells were detected in the passage 1 VEC culture. The separated CD146-positive cells showed stronger endothelial and decreased

mesenchymal properties compared to the unsorted cells in short term cultures. However, myofibroblast-like morphology re-appeared later when cells reached confluence. This suggests that even though cell sorting can efficiently separate VECs from a mixed population culture, a standard cell culture medium (i.e. AF-12) cannot maintain the endothelial properties in long term cultures. To preserve endothelial features and prevent mesenchymal differentiation, VECs typically require a specific culture medium containing growth factors favouring endothelial growth (Paranya et al., 2001; Wylie-Sears et al., 2011; Ci et al., 2013). The use of EBM instead of AF-12 resulted in a more consistent VEC phenotype, as evidenced by enhanced endothelial marker expression and decreased α-SMA and SMemb expression. However, EBM did not inhibit the proliferation of contaminating cell types. A combination of cell sorting and of a specific endothelial medium culture could improve the purity of VECs as well as maintain them for longer culture, however, further investigations are required to demonstrate it. For unsorted VECs, early passage (less than passage 3) and less confluent cells should be used for studies of VECs to guarantee a reasonable culture purity (Gould and Butcher, 2010).

Primary VIC cultures exhibited a fibroblast-like morphology, which is consistent with previous reports (Heaney et al., 2009; Waxman et al., 2012). However, some morphological variation was observed and a different response to enzymatic dissociation was observed with some cells showing stronger adhesion than others. This adherence difference has been associated with differential expression of α-SMA, with near 100% expression in more adhesive cells (Blevins et al., 2006). The heterogeneity in native VICs has been reviewed by Liu et al. (2007), who suggested that there are at least five sub-phenotypes. These sub-populations respond to stimulation and environmental changes differently and have different functions, but currently there is no definitive marker panel to identify each VIC sub-

phenotype. In general, VICs do not express VEC markers and are vimentin positive (Liu et al., 2007; Heaney et al., 2009). VICs are further confirmed by the expression of SMemb, SM22 and α-SMA, which are thought to indicate VIC activation (aVICs) (Della Rocca et al., 2000; Rabkin et al., 2001; Wiester and Giachelli, 2003; Disatian et al., 2008; Han et al., 2008; Stephens et al., 2011).

Recently aVICs have attracted some attention in the valvular research field, as they were found to play a key role in valve remodelling or stromal degeneration (Rabkin et al., 2001; Disatian et al., 2008; Gupta et al., 2009; Han et al., 2008). In diseased valves, there is an increase of α-SMA positive cells close to the endothelium, and these aVICs are presumed to contribute to valve damage and attempts at repair (Disatian et al., 2008; Han et al., 2008). Moreover, in vitro studies suggest that the aVIC population is dynamic, mechanosensitive and responsive to external stimulation. For example, culture conditions, such as cell density, substrate types and movement, can affect the level of α-SMA expression (Engler et al., 2006; Stephens et al., 2011; Xu et al., 2012). These findings may explain the discrepancy of α-SMA expression observed between the current study and a previous report (Taylor et al., 2000). Under culture conditions using three dimensional collagen substrates, the appearance of α-SMA positive cells suggests that a cell population analogous to the disease phenotype is present, but this can be reversed by mechanical treatment of the cells, reverting them to a more normal fibroblast phenotype (Waxman et al., 2012). Functional studies of mitral VICs are required. Cultured mitral VICs are capable of ECM synthesis and are involved in wound repair (Lester et al., 1993; Durbin et al., 2005; Flanagan et al., 2006; Fayet et al., 2007; Gupta et al., 2008). The effects of various conditions on canine valve cell phenotype and function requires further investigation if cultured VECs and VICs are to be used for modelling mitral valve disease.

Conclusion

The characterisation of canine mitral VECs and VICs demonstrated phenotypic heterogeneity under the static culture conditions used in the current study. These findings should contribute to improving the understanding of canine mitral valve cellular biology, and should benefit mitral valve disease research and tissue engineering studies.

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Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table 1. PCR sequences for PCR characterization of valve endothelial cells (VECs) and valve interstitial cells (VICs).

Gene	Primer Sequence (5'-3')	Product size
CD31	AATCCCAAATTCCACGTCAG GAATGGAGCACCACAGGTTT	346 bp
vWF1	CTGGGAGAAGAGAGTCACGG GTGGATGGAGTACACGGCTT	235 bp
vWF2	GGCTGTACCTGGATGAGAGG GACAGGACAGGCTCCTTTTG	228 bp
vWF3 (Fulton et al., 2000)	AATATAGGGCCCCGGCTCACTCAA ACATCCCCGGGCCTCTTCTCATTC	512 bp
SM22	AAGAACGGCGTGATTCTGAG CGGTAGTGCCCATCATTCTT	269 bp
α-SMA	GGGGATGGGACAAAAGGACA GCCACGTAGCAGAGCTTCTCCTTGA	525 bp
SMemb	AGAAGCGAGCTGGAAAACTG TCTTGCTCTGTCCGATTCTG	252 bp
Vimentin	GGAGCAGCAGAACAAGATCC AGACGTGCCAAAGAAGCATT	282 bp
GAPDH	CATCAACGGGAAGTCCATCT GTGGAAGCAGGGATGATGTT	428 bp

555
556 Table 2. Details of the antibodies used for canine mitral valve endothelial (VEC) and valve
557 interstitial cell (VIC) immunocytochemistry characterisation.
558

Primary antibody	**Species raised**	**Cat Number & Supplier**	**Dilution**
Anti-CD31	Rabbit polyclonal	Ab28364, Abcam, UK	1:200
Anti-SMemb	Rabbit polyclonal	Ab24761, Abcam, UK	1:400
Anti-vimentin	Mouse monoclonal	V6389, Sigma, USA	1:1600
Anti-α-SMA	Mouse monoclonal	A2547, Sigma, USA	1:400
Secondary antibody			
Anti-rabbit IgG (H+L) Alexa Fluor568	Goat	A11011, Invitrogen, UK	1:500
Anti-mouse IgG (H+L) Alexa Fluor488	Goat	A10667, Invitrogen, UK	1:500

559
560

Figures Legends

Figure 1. Canine mitral valve cell morphologies.

Upper Panel; primary isolated canine mitral valve endothelial cell (VEC) morphology. A. At the early stage, single colony clusters randomly appeared in the cultures. B. Once the cells reached confluence, the majority exhibited a cobblestone morphology and were growth-contact inhibited. Scale bar = 100 μm.

Lower Panel; primary cultured valve interstitial cell (VIC) morphology before confluence (C) and at confluence (D). Cells in VIC cultures exhibited fibroblast-like morphology, and tended to arrange in a 'hill and valley' pattern in confluent cultures. Scale bar = 200 μm.

Figure 2. Canine mitral valve endothelial cells (VECs) and valve interstitial cells (VICs) characterised by RT-PCR. Upper panel: VECs; the endothelial cell marker (CD31) and the activated VIC markers (α-SMA, SMemb and SM22) were detected in VECs. Vimentin and GAPDH were used as internal controls. Lower panel: VICs; cells were negative for the VEC marker CD31, but positive for α-SMA, SMemb, SM22 and vimentin. cDNA template derived from a VEC culture was used to amplify CD31 transcripts in the same experiment as a positive control (data not shown). Vimentin and GAPDH were used as internal controls.

Figure 3. Canine mitral valve endothelial cells (VECs) characterised by immunohistochemistry and DiI-Ac-LDL. Upper panel; DiI-Ac-LDL uptake by VECs in culture (A) and cell distribution illustrated by bright field microscopy (B). Scale bar = 100 μm. Lower panel; characterization of canine mitral VEC culture by immunofluorescence. Cell expressed CD31 (red in C), vimentin (green in G) and SMemb (red in K), but were negative for α-SMA (O). DAPI (blue) stained cell nuclei. DAPI channel (A, E, I and M), target protein channel (B, F, J and N) and merged images (C, G, K and O) are shown respectively for each marker. The same primary cultures cell lines served as negative controls (primary antibodies were omitted; D, H and L). Cells from a VIC culture showing expression of α-SMA served as a positive control (P). Scale bar = 50 μm.

Figure 4. Endothelial properties of canine mitral valve endothelial cells (VECs) under different culture conditions. EBM cultures (left panel) and AF-12 cultures of VECs (right panel) showing uptake of DiI-Ac-LDL (upper panel) and expression of CD31 (lower panel). There is a greater intensity of staining for EBM cultures. DAPI (blue) stained cell nuclei. Scale bar = 100 μm

Figure 5. Canine mitral canine mitral valve endothelial cells (VECs) under different culture conditions, characterised by semi-quantitative RT-PCR (arbitrary units compared to the reference gene GAPDH). Cells in EBM demonstrated higher expression of CD31 and decreased expression of α-SMA and SMemb compared to VECs and VICs cultured in AF-12.

Figure 6. Canine mitral valve interstitial cells (VICs) characterised by immunohistochemistry and DiI-Ac-LDL. Upper panel; lack of DiI-Ac-LDL uptake by VICs (A) and cell distribution illustrated by bright field microscopy (B). Scale bar = 200 μm. Lower panel; characterization of canine mitral VIC culture by immunofluorescence. Cells expressed vimentin (green in G), SMemb (red in K) and α-SMA (green in O), but were negative for CD31 (C). DAPI (blue) stained cell nuclei. DAPI channel (A, E, I and M), target protein channel (B, F, J and N) and merged images (C, G, K and O) are shown respectively for each marker. The same cell lines served as negative controls (primary antibodies were

607 omitted; H, L and P). Cells from a VEC culture showing expression of α-SMA served as a
608 positive control (D). Scale bar = 50 μm.
609

Figure1

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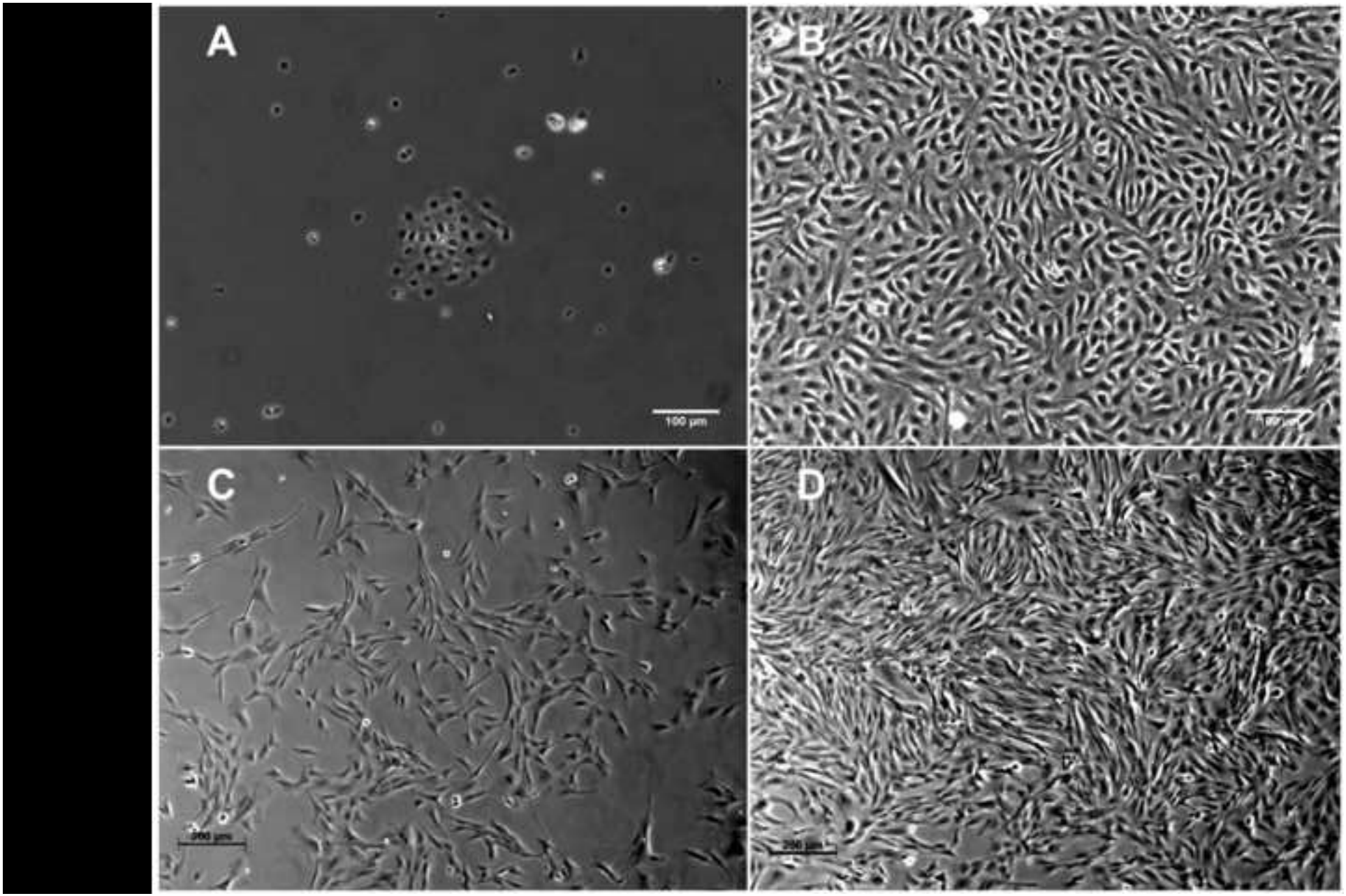

Figure2

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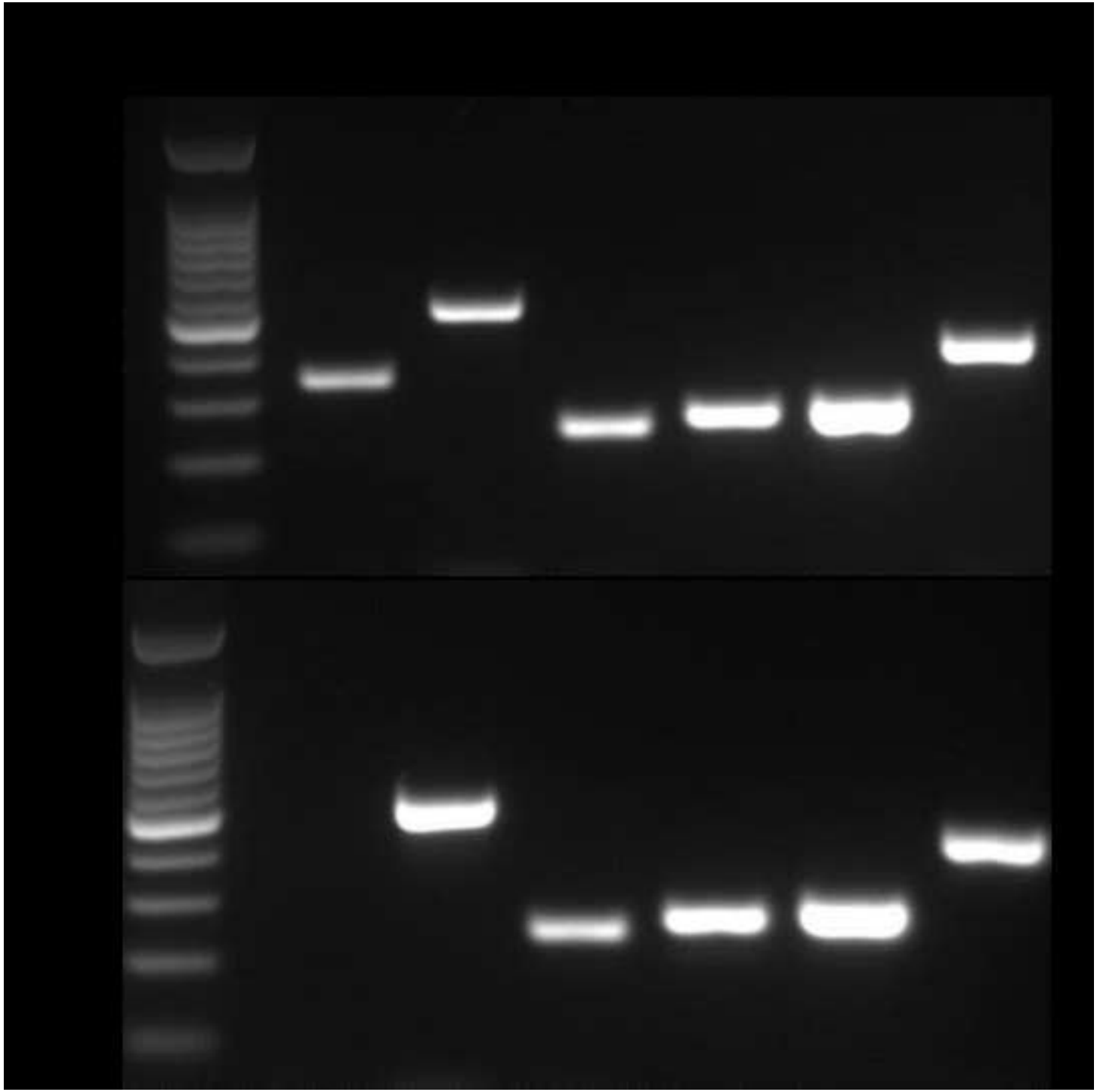

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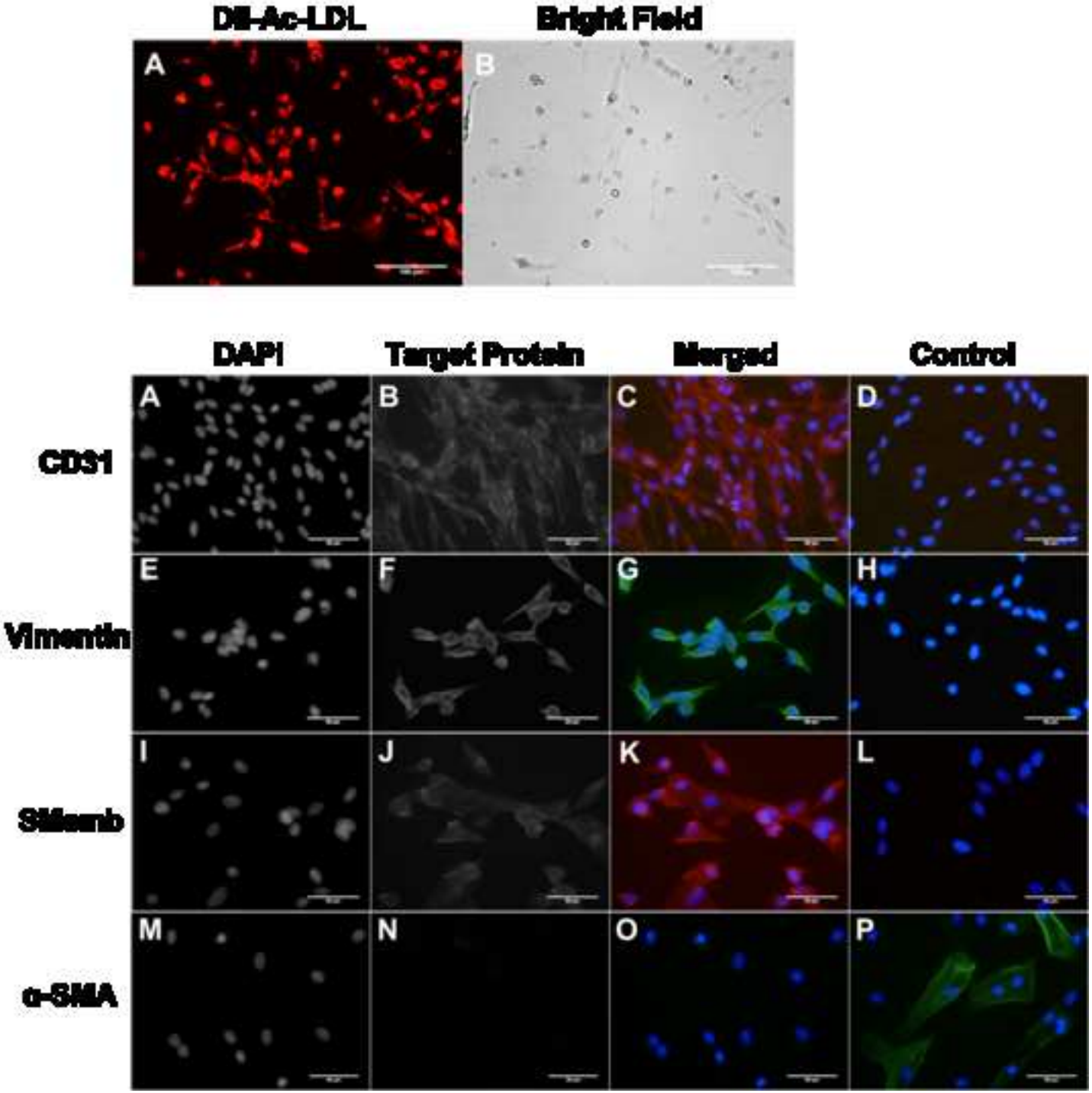

Figure4

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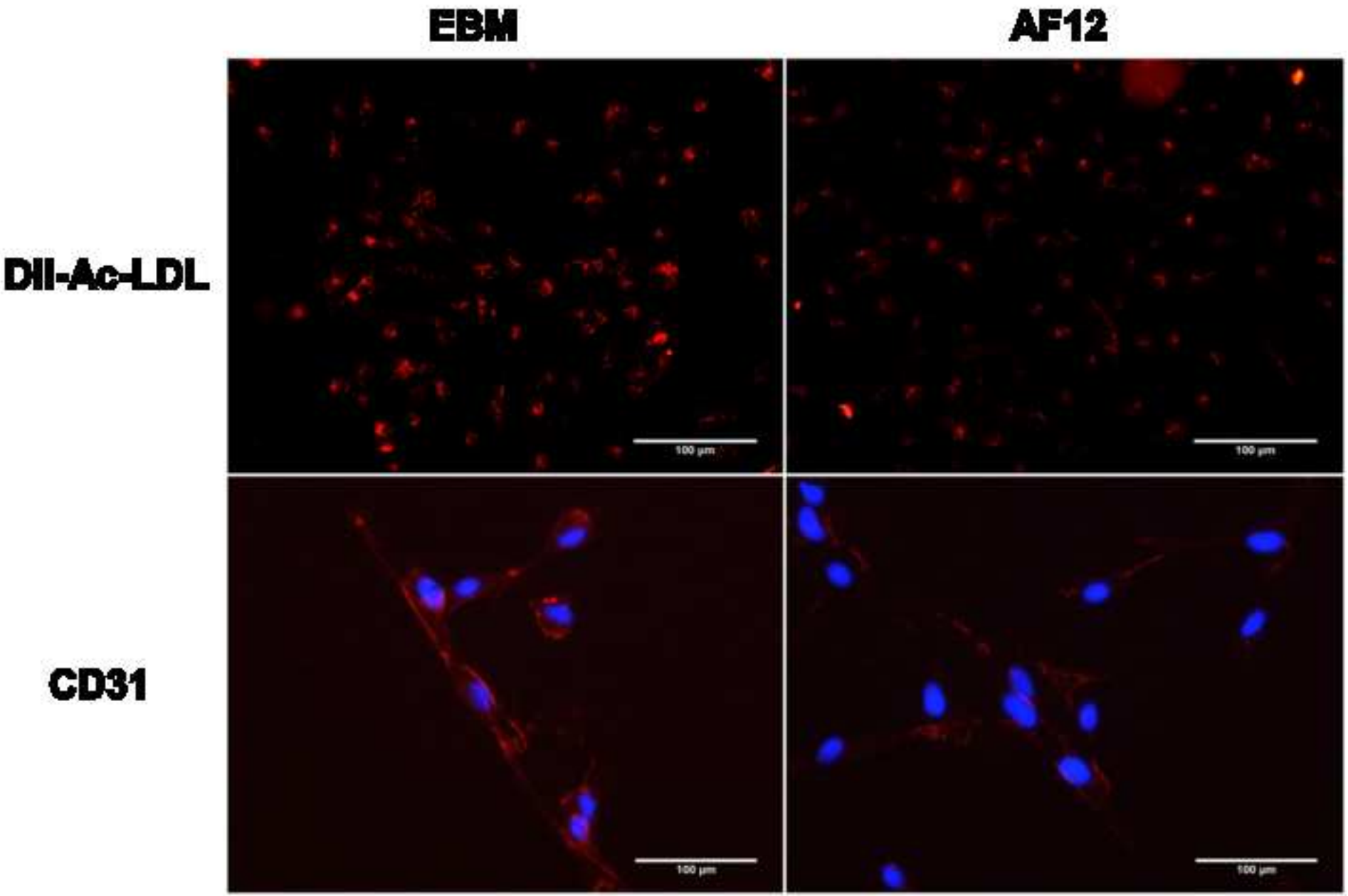

Figure5

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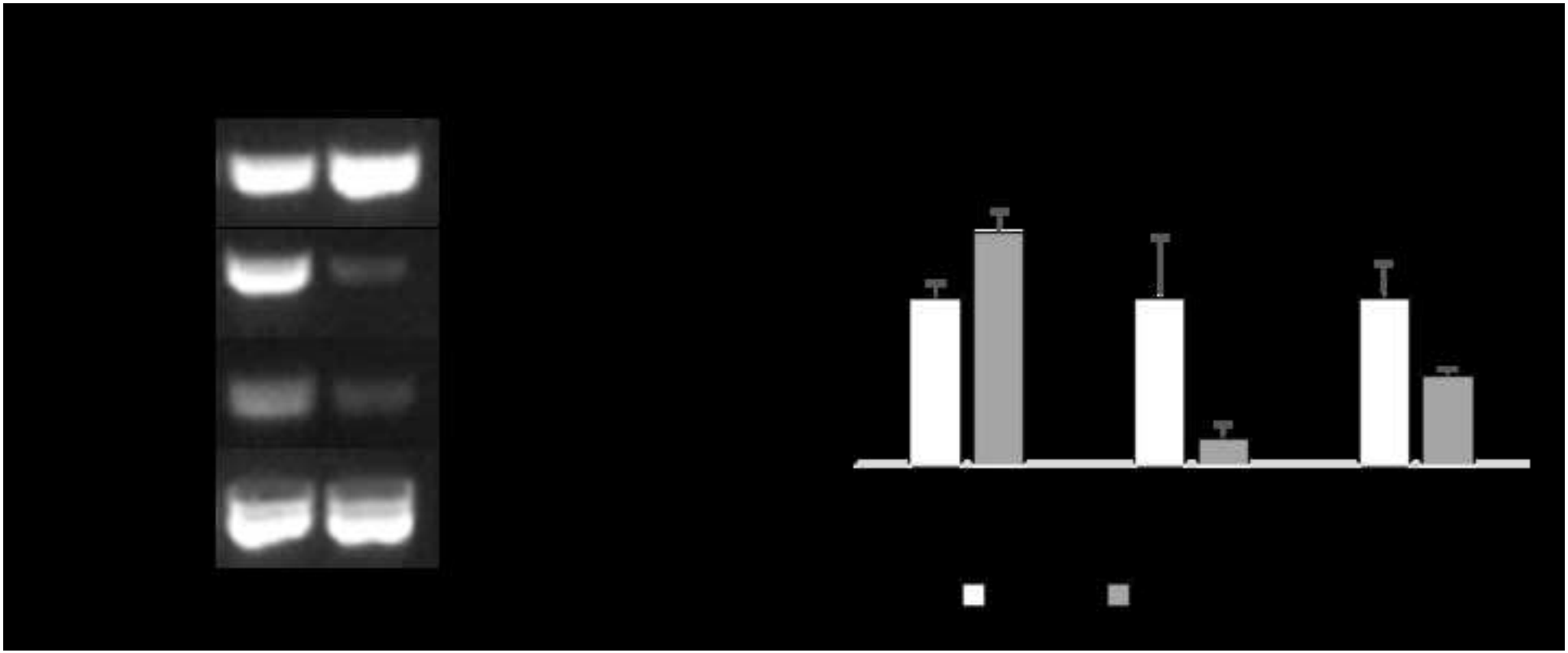

Figure6

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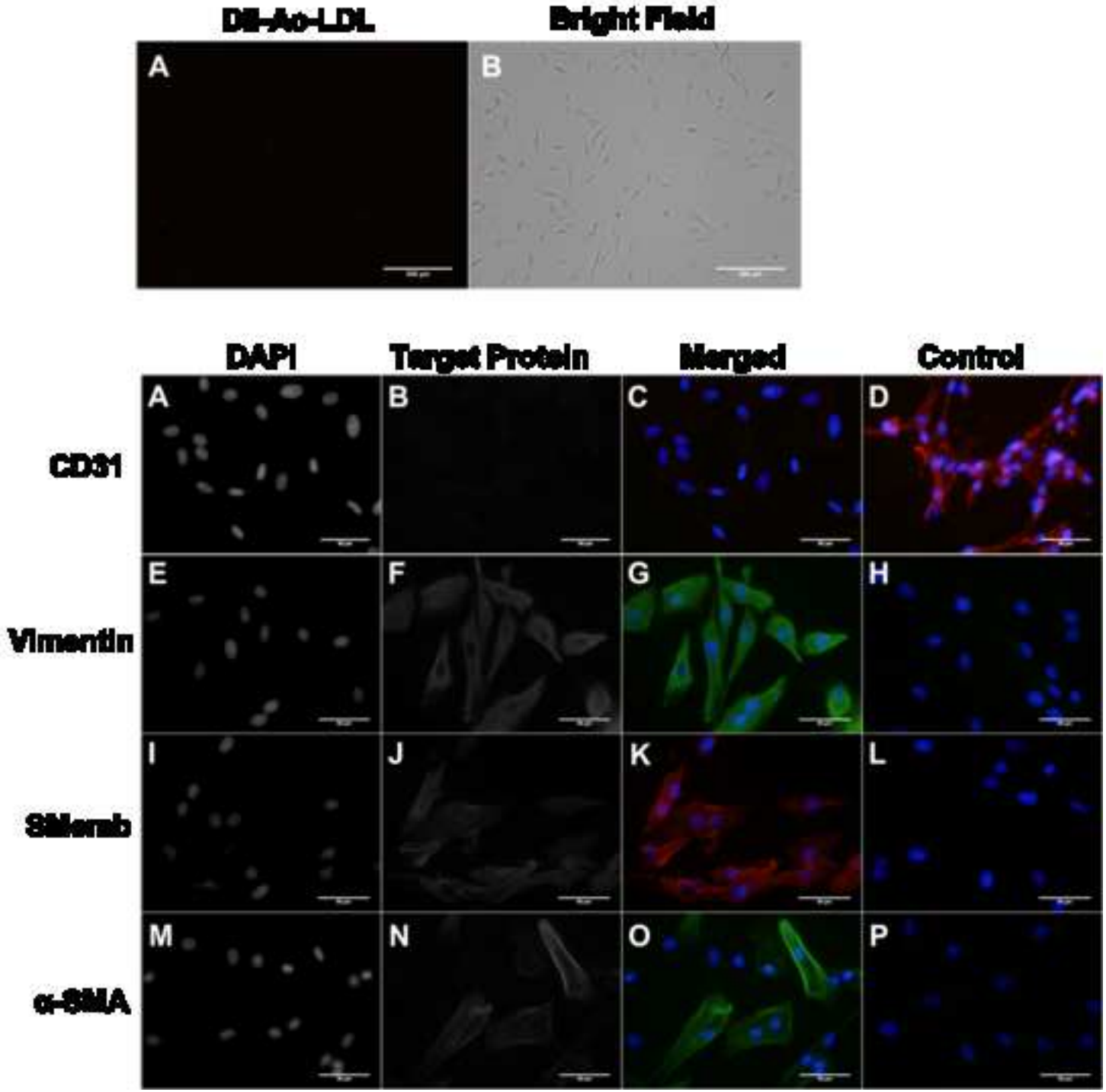

Figure 4

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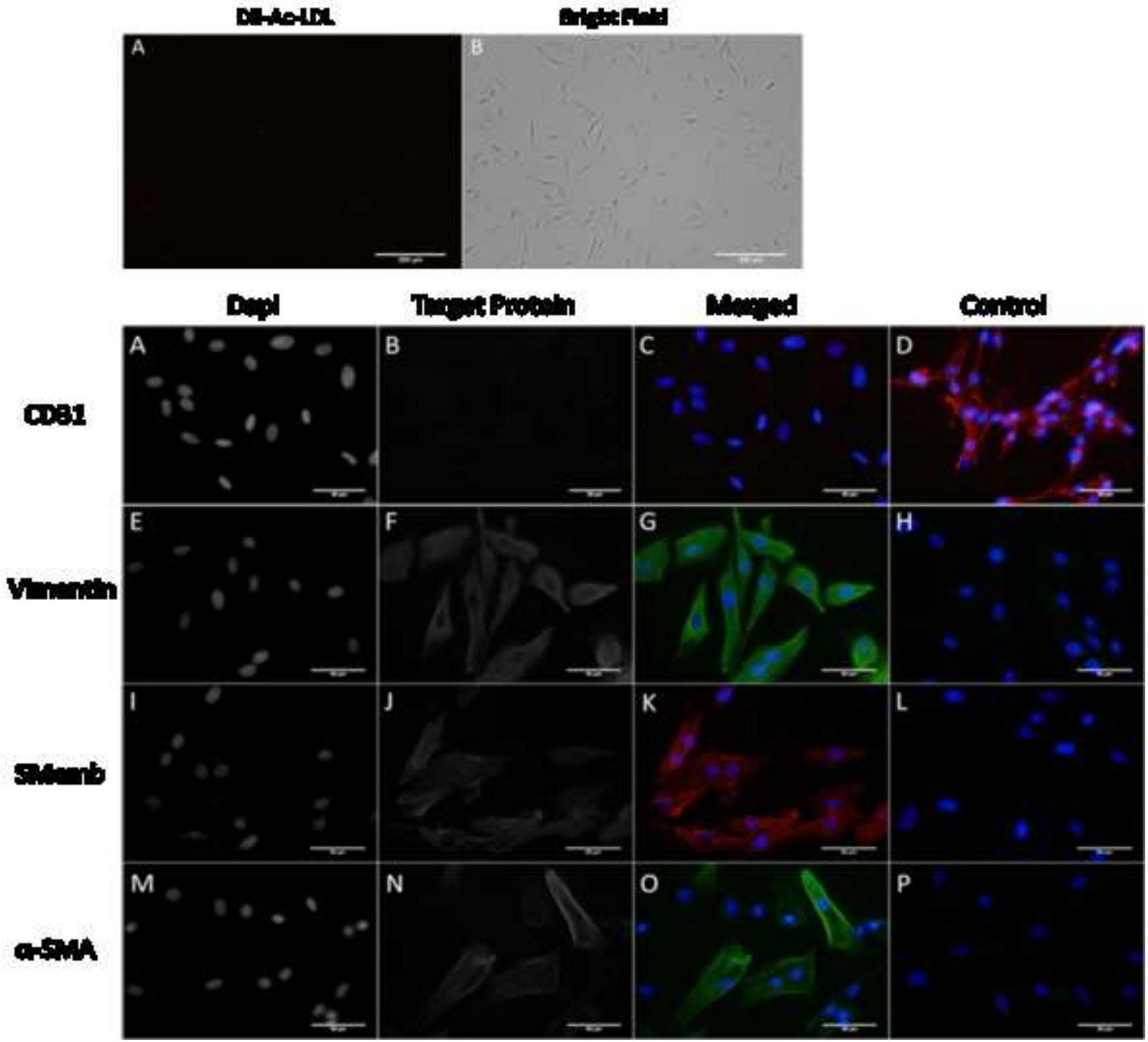

Figure 6

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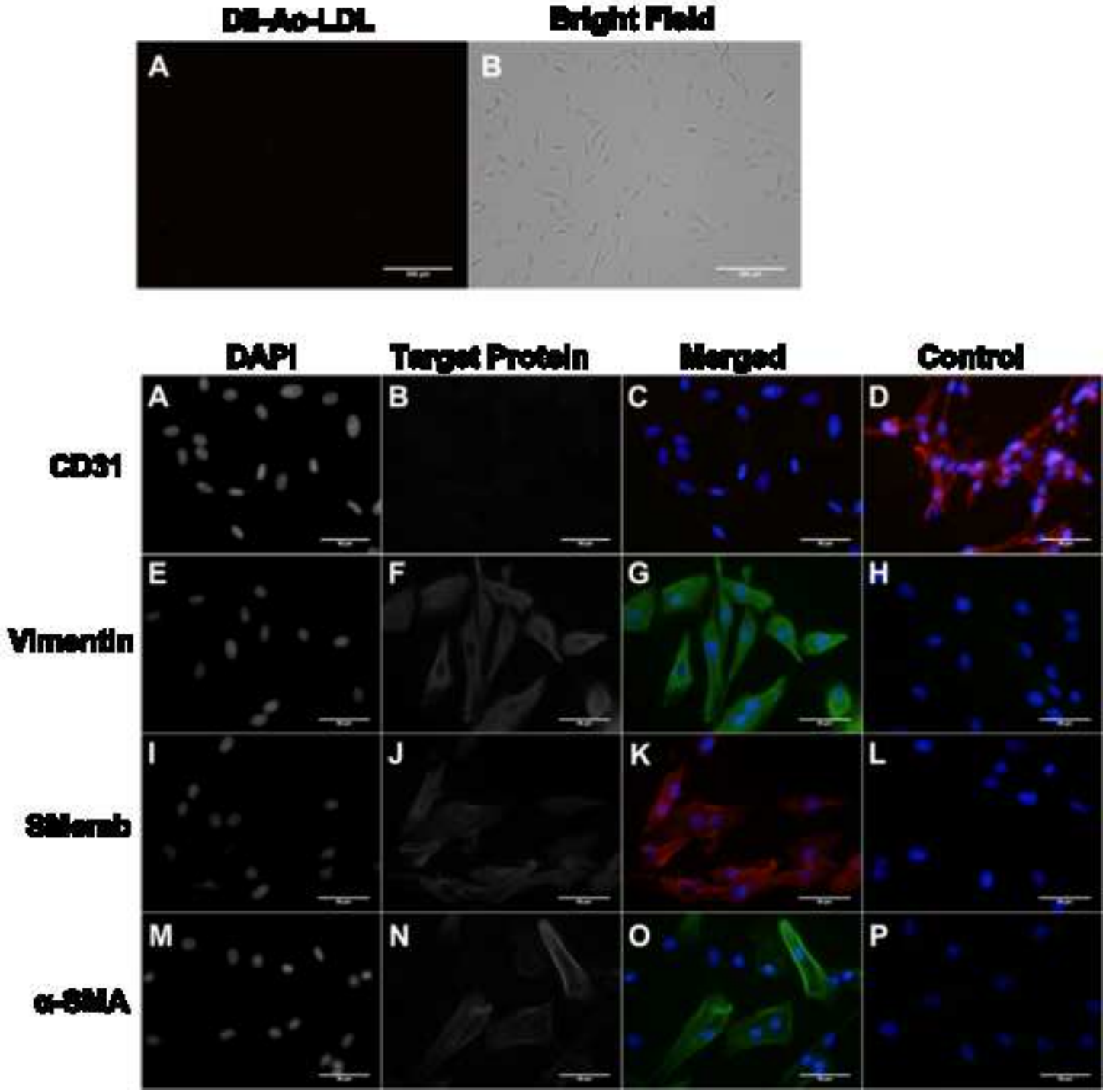

Highlights:

- Successful culturing and characterising of valve interstitial and endothelial cells from the same subject.
- First reported culturing of valve interstitial cells (VICs) that did not favour a migratory phenotype.
- First reported culturing of canine valve endothelial cells (VECs).
- Assessment of the value of multiple cell markers in cell phenotyping.
- Identification of CD31 expression and acetylated low density lipoprotein (Dil-Ac-LDL) uptake as the prime markers for differentiating VECs from VICs.
- Identification of phenotypic plasticity under culture conditions and optimal passage for attaining the purest VEC population.

Highlights:

1. Successful culture and characterisation of valve interstitial and endothelial cells
2. Identification of CD31 expression and Dil-Ac-LDL uptake as the prime markers for differentiating VECs from VICs.
3. A specific endothelial culture medium for primary VEC cultures enhanced the endothelial properties of the cells

***Revision Note**

We would like to thank the reviewers for their comments. There were instances where the line numbers quoted did not match those of the current version of the manuscript, but we think we have managed to respond to each comment in turn.

Reviewer 1

Point 1: "The authors are not clear on whether they were able to culture a pure VEC population that then subsequently differentiated (e.g. via EndMT) or whether the population was heterogeneous (VEC+VIC) to start with. Their aSMA data suggests the latter, but if they combined with IHC of normal canine MV, it might support that canine VEC possess aSMA expression in vivo."

Response: Indeed, sub-endothelial VIC contamination in primary VECs isolated by enzymatic digestion has been recognised as a common technical issue (Cheung et al., 2008; Hoerstrup et al., 1998). However, we cannot completely rule out the possibility of cell differentiation in culture. Firstly, the α-SMA expression in VECs was mainly identified at gene level and it was almost absent at protein level. It is difficult to tell whether the α-SMA gene expression was from differentiating VECs or contaminating cells or from both. Secondly, which is probably more robust evidence, is the extensive expression of activated mesenchymal marker SMemb expression in the VEC cultures. This marker has been found highly expressed in embryonic VECs during development or in activated VICs during valve disease processes, while in healthy adult heart valves, the expression has been described as absent/minimally expressed (Aikawa et al., 2006). Therefore, we propose that the canine mitral VECs we cultured in vitro may possess some embryonic differentiation potentials and we have addressed the above points in the discussion of the revised manuscript.

With regards to native valve α-SMA expression, we did look at it in normal canine MV by immunofluorescence (data not shown); the expression was very limited and mainly restricted to atrialis, and this finding is consistent with our own and others previous reports (Han et al., 2008; Disatian et al., 2008).

Point 2: "Their method is largely similar to that of Gould et al (JoVE 2012), which raises the question of whether ECM coating is essential for VEC adhesion. It would be good to know, as that can be a real problem out of the gate for others."

Response: From our experience, the answer is no. We once accidentally cultured our VECs on polypropylene flasks without gelatin coating; the cells still adhered and proliferated, however, with a different morphology and the efficiency of adherence was not investigated (data not shown). Indeed, it has been reported that additional ECM coating to plastic culture flasks can enhance endothelial cell adherence, proliferation and affect endothelial cell morphologies (Relou et al., 1998; Schelling et al., 1988; Xia et al., 2011). Both gelatin and collagen are commonly used for valve endothelial culture in previous reports (Wylie-Sears et al., 2011; Gould and Butcher, 2010). For these reasons, we would recommend matrix coating for endothelial culture.

Point 3: "Also, it isn't clear from my reading of the manuscript whether the VEC cultured in this study ended up being suitable for follow-on experiments on VEC behavior. Are enough cells available at passage 3 without compromising homogeneity?

Response: The answer is yes. Primary VECs P0 yields to $0.66 \times 10^6 - 3.5 \times 10^6$ depending on dog size and valve leaflet collected(anterior/posterior or both), and on average 1.5×10^6 approximately. This can further yield to approximate 1.0×10^7 at P1, and around 1×10^8 at P2. This number should be sufficient to do routine experiments such as immunocytochemistry (usually 1×10^4 cells per sample)

and RT-PCR (usually 0.5-1 x 10^6 cells for RNA extraction). In terms of cell purity, according to our findings, VECs cultured in AF-12 medium (without additional purification or endothelial medium replacement) showed at least 50%-60% typical endothelial cobblestone morphology within 3 passages. We didn't do endothelial phenotypic quantification on immunocytochemistry, but we presumed the actual number would be higher than morphological observation, as CD31 or DiI-Ac-LDL positive cells were heterogeneous in morphology (not limited to cobblestone shape only).

Point 4: "The authors suggest EBM culture can improve EC phenotype homogeneity at a sacrifice of proliferation, but does this approach create enough cells for an experimental assay?"

Response: Yes. When used EBM for primary P0 VECs culture, there was cell apoptosis and endothelial colony disappear during the early stages, but the cells started to proliferate rapidly in later culture periods. For those VECs experiencing EBM switch at P1 or P2, they did not show dramatically decreased proliferation compared to AF-12 culture. To avoid confusion the P0 EBM finding has been removed from the manuscript.

Point 5: "Do the authors recommend using EBM during whatever treatment would be performed, or switching to AF-12 Media?"

Response: Once starting culture the VECs in EBM we would continue with EBM rather than switching to AF-12. For experiments specifically investigating VEC behaviour and function, we would recommend to use EBM rather than AF-12, as the former is more optimal in maintaining VEC phenotype.

Point 6: "As a minor but important comment, having standard deviation bars on Figure 6 would be helpful to assess the expected variation in isolation quality and/or subsequent culture performance of the phenotypes of these cells."

Authors' Response: We've added standard error bars on Figure 5 (original Figure 6) in the revised manuscript (n=3).

Reviewer 2

Point 1: "Not sure about numbering of subsections in M and M and in Results - what is editorial policy?"

Response: We've removed the numbering of subsections in the revised manuscript.

Point 2: "Line 88 - presumably valves showed no gross evidence of MMVD?"

Response: We've addressed this point in the revised manuscript; please see Line 84-85.

Point 3: "Line 102 - a bit more pre-amble on the media experiments would be useful, just to put them into context. Typo – passage (P1)."

Response: We've addressed this point in the revised manuscript; please see Line 109-121.

Point 4: "Line 110 - might be useful to add a third sub-heading on cell passage (see later comment)."

Response: We have addressed this point in Line 104-107 in the revised manuscript.

Point 5: "Lines 117, 135 and 149 - cells from which passage were used on these experiments - was this consistent?"

Response: We have addressed this point in Line 112, 200 and 222 in the revised manuscript.

Point 6: "Lines 137 and 149 - details of slides - chamber slides mentioned."

Response: We have addressed this point in Line 147-148 in the revised manuscript.

Point 7: "Line 164 - was detected by RT-PCR in VEC culture?"

Response: We have addressed this point in Line 201-202 in the revised manuscript.

Point 8: "Line 177 - was detected by RT-PCR for….?"

Response: We have addressed this point in Line 223-224 in the revised manuscript.

Point 9: "Move section on effect of culture medium (lines 182-196) to before section on VIC morphology and characterization to be consistent with Methods."

Response: We have moved this section as requested; see Line 214-220.

Point 10: "Line 194 - typo - EBM.Both…"

Response: We have addressed this point in the revised manuscript.

Point 11: "Line 201 - Waxmann reference not in references."

Response: We have addressed this point Line 534 in the revised manuscript.

Point 12: "Line 239 - ..although it did not preserve…?"

Response: We have addressed this point in the revised manuscript.

Point 13: "Line 256 - Liu et al (2007),…"

Response: We have addressed this point in the revised manuscript.

Point 14: "Line 265 - Han 2013 - a or b?"

Response: We cannot locate this comment in the original manuscript. Effectively has been resolved

Point 15: "Several references in reference list are incorrect or incomplete - e.g. lines 381 and 386, Paranya reference has a space in it. There is variability in how journals are cited."

Response: We have addressed these inconsistencies and errors in the reference section of the revised manuscript.

Point 16: "Images - quality of text on images is poor."

Response: We have improved the text quality in the revised manuscript.

Point 17: “Figure 3 - top panel association of positive staining with cells shown in the bright field image is not as convincing as the other images.”

Response: We have checked the image and can confirm that the bright field image is correctly matched with the fluorescence image. However, the focusing of the bright field image was not optimal. We have tried to enhance that by adjusting brightness and contrast of the bright field image in the revised manuscript.

Point 18: “Figures 3 and 4 - for clarity legends should explain column titles - what do the target protein images show, what is the merged image.”

Response: We have addressed this point in the revised manuscript. Please see legends of Figure 3 and 6 (Figure 4 in original manuscript).

***Revision Note2**

Comments E Bloome

1. The current Figure 4 is a copy of Figure 6, I think Figure 4 should be what was Figure 5 in the previous submission.

Figure identities seem correct; will up-load fresh set of figure images.

2. Cannot find Bischoff (2001) reference in the text.

No Bischoff 2001 in the text or reference list.

Comments A. Philby

1. Please check author's initials, e.g. "D.A. Argyle".

Changed to D.J.

2. Some reformatting of the Corresponding author's details will be required.

Not sure what is required.

3. Most non-standard abbreviations should be defined when first used in the Abstract and again in the Main text. Abbreviations that do not need to be defined include PCR, ELISA, DNA and RNA. In the Abstract, please change "reverse transcription polymerase chain reaction (RT-PCR)" to "reverse transcription PCR".

MS checked and corrected

4. Groups of references cited in the text should be arranged chronologically according to the idiosyncratic journal style, e.g. "Aupperle et al., 2009; Aupperle et al., 2010; Black et al., 2005; Hadian et al., 2007; Han et al., 2010; Han et al., 2013b" should be cited as "Black et al., 2005; Hadian et al., 2007; Aupperle et al., 2009, 2010; Han et al., 2010, 2013b".

Corrected; EndNote seems to think TVJ format is different!

5. Most units should be separated from values by a space, although percentages should be written without a space. Similalry, when writing temperatures, the value should be separated from the symbol for degrees Celsius by a space, e.g. "37°C" should be written as "37 °C" using the Word symbol for degrees Celsius rather than a superscripted letter "o".

MS checked and corrected

6. Some reformatting of tables and figure legends will be required.

Not sure what is required?

7. Please check spacing and punctuation throughout the manuscript.

Done.

8. Please reformat the Highlights with bullet points and define abbreviations. Each Highlight should be no more than 125 characters including spaces.

Done.

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29/7/2014

Please find attached "Canine Mitral Valve Interstitial and Endothelial Cell Culture and Characterisation" for consideration for publication in TVJ.

The paper describes the culturing of valve interstitial and endothelial cells from canine mitral valves. This is important as there is increasing interest in using culture systems to investigate mechanisms of pathogenesis in canine mitral valve disease, not least because of the difficulties in sourcing native valves.

No previous study has reported on canine VECs, which are much harder to grow than VICs, and previous reports of VICs have used techniques that only cultured migratory phenotypes.

This study provides the basic information necessary for accurate VIC and VEC culture and the techniques required for accurate phenotyping.

Brendan Corcoran

Brendan Corcoran